

ORIGINAL ARTICLE

Analysis of subcellular localization of Myo7a, Pcdh15 and Sans in *Ush1c* knockout mice

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Summary

Usher syndrome (USH) is the most frequent cause of combined deaf-blindness in man. An important finding from mouse models and molecular studies is that the USH proteins are integrated into a protein network that regulates inner ear morphogenesis. To understand further the function of harmonin in the pathogenesis of USH1, we have generated a targeted null mutation *Ush1c* mouse model. Here, we examine the effects of null mutation of the *Ush1c* gene on subcellular localization of Myo7a, Pcdh15 and Sans in the inner ear. Morphology and proteins distributions were analysed in cochlear sections and whole mount preparations from *Ush1c*^{-/-} and *Ush1c*^{+/-} controls mice. We observed the same distribution of Myo7a throughout the cytoplasm in knockout and control mice. However, we detected Pcdh15 at the base of stereocilia and in the cuticular plate in cochlear hair cells from *Ush1c*^{+/-} controls, whereas in the knockout *Ush1c*^{-/-} mice, Pcdh15 staining was concentrated in the apical region of the outer hair cells and no defined staining was detected at the base of stereocilia nor in the cuticular plate. We showed localization of Sans in the stereocilia of controls mouse cochlear hair cells. However, in cochleae from *Ush1c*^{-/-} mice, strong Sans signals were detected towards the base of stereocilia close to their insertion point into the cuticular plate. Our data indicate that the disassembly of the USH1 network caused by absence of harmonin may have led to the mis-localization of the Protocadherin 15 and Sans proteins in the cochlear hair cells of *Ush1c*^{-/-} knockout mice.

Keywords

deafness, inner ear, knockout mouse, Usher 1C, Usher syndrome

Introduction

Usher syndrome (USH) is an autosomal recessive disorder characterized by congenital hearing loss and progressive retinal degradation leading to gradual loss of the visual field and blindness.

Three major clinical subtypes (USH type I, USH type II and USH type III) are distinguished on the basis of differences in the severity of the hearing loss, the presence or absence of vestibular dysfunction and the age of onset of retinitis pigmentosa (RP) (Smith *et al.* 1994). In USH type 1, the hearing loss is profound and vestibular function is absent. The onset of progressive RP is before puberty. Usher syndrome type 2 is associated with less severe deafness, normal vestibular function and onset of RP during or after puberty. Usher syndrome type 3 patients also have milder

deafness, but, unlike in USH2, the hearing loss is progressive, there is variable impairment of vestibular function and late-onset RP. Each USH subtype is genetically heterogeneous. To date, seven USH1 loci (USH1B-USH1H) have been identified by linkage analyses of USH1 families. Five of the corresponding genes have been cloned: the actin-based motor protein myosin VIIa (*Myo7a*, *USH1B*) (Gibson *et al.* 1995; Weil *et al.* 1995); two cadherin-related proteins, otocadherin or Cadherin 23 (*Cdh23*, *USH1D*) (Bolz *et al.* 2001; Bork *et al.* 2001) and Protocadherin 15 (*Pcdh15*, *USH1F*) (Ahmed *et al.* 2001; Alagramam *et al.* 2001a); and two scaffold proteins, harmonin (*USH1C*) (Verpy *et al.* 2000; Bitner-Glindzicz *et al.* 2000) and Sans (*USH1G*) (Kikkawa *et al.* 2003; Weil *et al.* 2003). The USH proteins are involved in hair bundle morphogenesis in the inner ear by means of protein-protein interactions. In a combination of cell cotransfection

and *in vitro* binding assays, harmonin has been shown to bind to any of the other USH proteins (El-Amraoui & Petit 2005; Yan & Liu 2010; Zheng *et al.* 2010).

A mouse mutant has been reported for each of the known *Ush1* genes; shaker1 (*sh1*) for *Myo7a* (Gibson *et al.* 1995), waltzer (*v*) for *Cdh23* (Di Palma *et al.* 2001; Wilson *et al.* 2001), Ames waltzer (*av*) for *Pcdh15* (Alagramam *et al.* 2001b), deaf circler (*dfcr*) and targeted mouse models for *Ush1c* (Johnson *et al.* 2003; Lentz *et al.* 2007; Lefevre *et al.* 2008; Tian *et al.* 2010) and Jackson shaker (*js*) for *Ush1g* (Kikkawa *et al.* 2003). All of these mice are deaf, exhibit vestibular dysfunction and display similar morphological abnormalities in hair bundle development. In all of these models, the hair cell stereocilia vary irregularly in height and splay out from one another indicating defective lateral interactions. Investigations into the localization of the USH proteins within the developing stereocilia in mice, combined with *in vitro* studies to determine the various interactions between the constituent molecules, have revealed an 'Usher interactome' that is responsible for bundle cohesion. Some of the *Ush1* mutant mice (*sh1*, *v*, *av*) exhibited electroretinogram anomalies (Libby & Steel 2001), a defective retinal pigment epithelium has been described in *sh1* mice (Gibbs *et al.* 2003, 2004) and retinal degeneration has been reported in *Ush1c216AA* knockin mice (Lentz *et al.* 2010).

The gene encoding harmonin consists of 28 coding exons, alternative splicing of which leads to 10 USH1C isoforms. These alternative transcripts form three subclasses (a, b and c) depending on the domain composition of the protein. The isoform 'a' transcript subclass is expressed ubiquitously in many tissues, whereas the longest 'b' transcript is restricted largely to the inner ear. The short isoform 'c' has a much broader tissue distribution. The harmonin isoforms differ in the number of protein-protein interaction domains (PDZ, postsynaptic density/disc-large/zonal occludens 1), coiled-coiled domains (CC) and the presence of a proline-serine-threonine-rich domain (Verpy *et al.* 2000). Deaf circler, *dfcr* and *dfcr-2J* spontaneous mutant mice have been described as models for human USH1C. The mutant *dfcr* is defective in all harmonin isoforms (a, b and c). Only the harmonin b isoform subclass is affected by the *dfcr-2J* mutation (Johnson *et al.* 2003). However, altered harmonin isoforms may retain partial function because the normal reading frame of the *Ush1c* transcripts is not changed in the shortened *dfcr* transcripts of either isoform a or isoform b. Furthermore, none of the three PDZ-encoding domains are deleted in *dfcr* mutant transcripts. Both a USH1C knockin and knockout mouse have also been reported (Lentz *et al.* 2007; Lefevre *et al.* 2008). To further understand the role of harmonin in the pathogenesis that leads to USH1, we have recently generated a targeted null mutation *Ush1c* mouse model in which the first four exons of the *Usher 1c* gene have been replaced by a reporter gene (Liu *et al.* 2005; Yan *et al.* 2006; Tian *et al.* 2010). Our model is unique because none of the previous targeted mouse models for USH1C include a reporter gene in the construct to facilitate expression analysis in various tissues. Here, we examine the effects of *Ush1c*

mutation on spatial subcellular localization of Myo7a, Pcdh15 and Sans proteins in the inner ear. In whole mount of inner ears from mutant, Myo7a was not affected at the timepoint we analysed the mutant mice, although it is a critical part of the USH interactome. However, we found both Pcdh15 and Sans displayed an altered localization in the mutant mice that may have resulted from disruption of the entire USH1 complex.

Materials and methods

Inner ears isolated from the *Ush1c*^{-/-} and *Ush1c*^{+/-} mice at postnatal day 21 (PD21) were fixed by immersion in 4% paraformaldehyde (pH 7.4) for 2–5 h at 4°C. The organ of Corti was dissected from the cochlear spiral in phosphate-buffered saline (PBS) using a fine needle. Samples were then permeabilized in 0.5% Triton X-100 for 30 min, then washed in PBS. Non-specific binding sites were blocked using 5% normal goat serum (Life Technologies, Gaithersburg, MD, USA) and 2% bovine serum albumin (ICN, Aurora, OH, USA) in PBS for 2 h. Samples were incubated for 2 h in the primary antibodies at 5 µg/ml in blocking solution. After several rinses in PBS, samples were incubated in Alexa Fluor 488-conjugated anti-rabbit IgG goat at 1:400 (Molecular Probes, Eugene, OR, USA) for 40 min. Samples were mounted using a ProLong Antifade kit (Molecular Probes) and analysed with a laser scanning confocal microscope (LSM-510; Zeiss, Thornwood, NY, USA). The polyclonal antibody against Myo7a (ab3481) was obtained from Abcam (Cambridge, MA, USA). The anti-PCDH15 antibody was generated against a mixed peptide sequence corresponding to amino acid 24–37 (SWGQYDDDWQYEDC) and amino acid 1847–1860 (C+TFTTQPPASNPQWG), and the anti-USH1G antibody was against the central portion of the Sans protein (amino acid 354–372).

Results

Homozygous mutant mice (*Ush1c*^{-/-}) exhibit the abnormal behaviour (circling and head-tossing) that are typical of mice with profound hearing loss and vestibular dysfunction. *Ush1c*^{-/-} mice were completely deaf, as there was no detectable auditory-evoked brainstem response (ABR) with 100 dB SPL stimuli, whereas age-matched *Ush1c*^{+/-} controls showed ABR thresholds in the normal hearing-range at PD15 and PD22. Examination of hair cell surface preparations by scanning electron microscopy from birth (PD0) to PD120 in *Ush1c*^{-/-} showed progressively disorganized outer hair cell (OHC) stereocilia compared with the well-organized pattern and rigid structure typical of normal stereocilia. Stereocilia of inner hair cells (IHCs) of mutant mice also exhibited a disorganized appearance, but to a lesser degree than did the OHCs (Tian *et al.* 2010).

To address the possibility that absence of harmonin disrupts the USH1 protein complex, we analysed in this study the distribution of Myosin VIIa protein in whole mounts, and of Protocadherin 15 and Sans in cross sections, of inner

ears from *Ush1c*^{-/-} mice. Light microscopy examinations of sections through apical regions of the cochleae of *Ush1c*^{-/-} at PD21 revealed no apparent hair cell degeneration (data not shown). However, in cochlear whole mounts from *Ush1c*^{-/-} mice at PD21, some gaps are seen in the regular array of hair cells. Although the single row of IHCs and the three rows of OHCs can be distinguished by surface scanning of the hair cells (Figure 1b), fragmentation of the OHC stereociliary bundles into two clumps was observed, instead of an integral, single 'V'-shaped bundle as in wild-type hair cells (Figure 1d, arrow). This fragmented aspect was not detected in stereocilia of IHCs at this timepoint (IHC; Figure 1b), suggesting that they were beginning to degenerate. However, confocal microscopic analysis of the hair cells in the basal

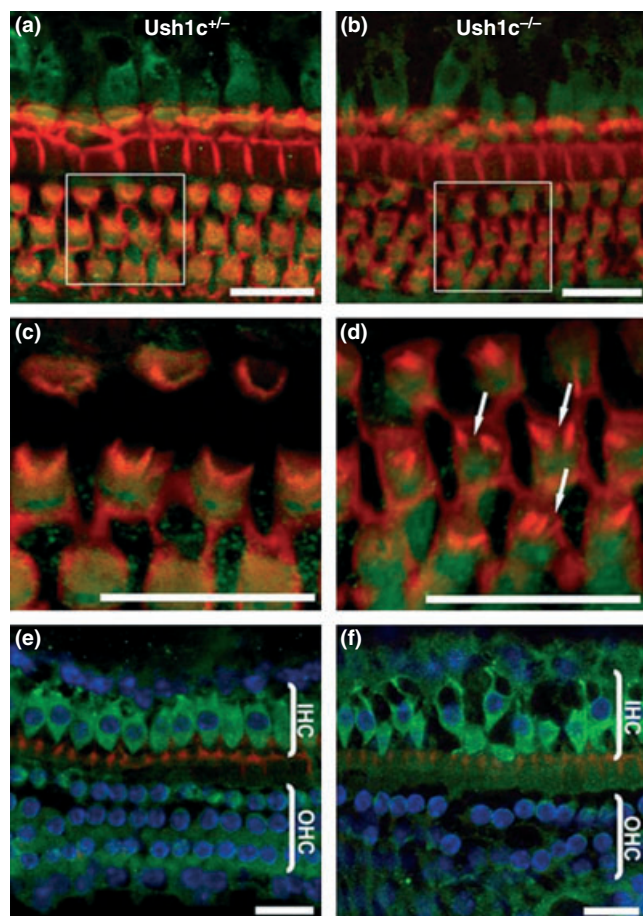


Figure 1 Abnormalities at the apical surface of outer hair cells (OHC) and structural defects of the hair cells in *Ush1c*^{-/-} mice at PD21. Cochlear whole mounts were stained with phalloidin (red) to reveal F-actin in stereocilia and an antibody against myosin 7a (green) to show the basal structure of the hair cells in *Ush1c*^{+/+} (a, c and e) and *Ush1c*^{-/-} mice (b, d and f). Stereocilia defects were observed in the middle part of the stereocilia bundles of the OHCs (d, arrows) that were not detected in stereocilia of inner hair cells (IHC; b). c and d show magnified images corresponding to the boxed areas in a and b respectively. The confocal analysis at the basal level of hair cells with nuclear staining (e and f) (DAPI, Blue). Bars = 20 μ m.

layer revealed structural morphological abnormalities in both OHCs and IHCs, with a more disorganized appearance in IHC (Figure 1f). Scanning electron microscopy of *Ush1c*^{-/-} mice from PD21 to PD120 showed a progressive degeneration of the hair cells and stereocilia of the cochlea (Tian et al. 2010). Myosin 7a has previously been shown to be expressed within the stereocilia and within the cuticular plate, which anchors the base of each stereocilium. In the present study, Myosin 7a was distributed throughout the cytoplasm in *Ush1c*^{-/-} and control mice in the labelled hair cells, revealing structural morphological abnormalities characterized by disorganized, misaligned inner and OHCs (Figure 1e, f).

In cochlear hair cells from heterozygous *Ush1c*^{+/-} control mice, we detected Protocadherin 15 at the base of stereocilia and in the cuticular plate (as shown by the arrow in Figure 2c), whereas in the mutant *Ush1c*^{-/-}, Protocadherin 15 immunoreactivity was found accumulated in the apical region of the OHC and no defined staining was detected at the base of stereocilia and little Pcdh15 expression was present at the cuticular plate (Figure 2d). Likewise, in the knock-out mice, Sans was undetectable in the stereocilia bundles of cochlear hair cells at PD21 (Figures 3b, d), in contrast to the heterozygous controls (*Ush1c*^{+/-}, Figure 3c). Instead, strong Sans staining was observed towards the base of stereocilia close to their insertion point into the cuticular plate with a slight staining of the cytoplasmic region of OHC in cochleae from *Ush1c*^{-/-} mice (Figure 3d). These results suggest a mis-localization of the Pcdh15 and Sans proteins in

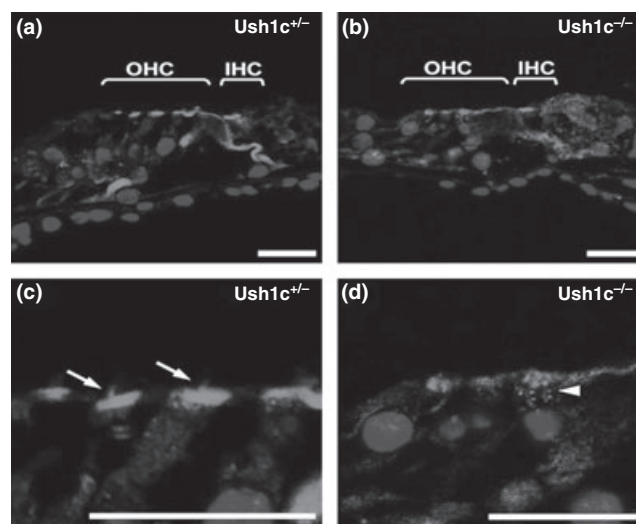


Figure 2 Localization of Protocadherin 15 (green) in the cochlear hair cells of *Ush1c*-knockout mice at PD21. Cross sections of the organ of Corti were stained with an antibody to Protocadherin 15 (green). In *Ush1c*^{+/+} mice (a and c), expression of Protocadherin 15 was localized at the base of stereocilia (left panel, arrow) and in the cuticular plate. In contrast, in *Ush1c*^{-/-} mice (b and d), Protocadherin 15 immunoreactivity appears (arrowhead) diffuse above nuclei (DAPI-Blue). Bars = 20 μ m.

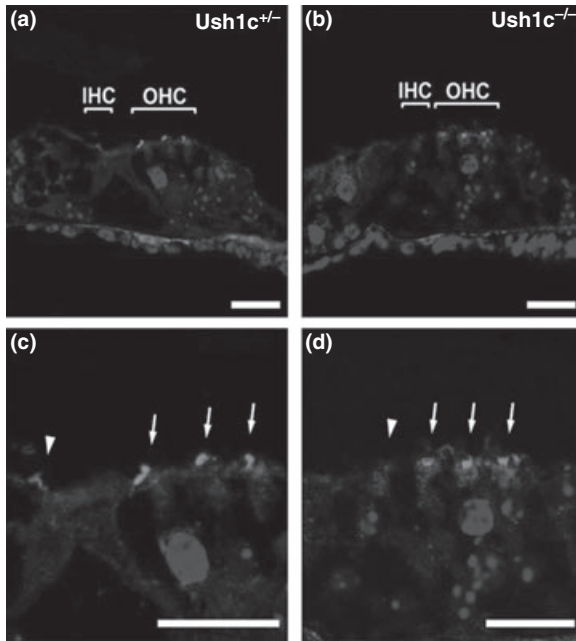


Figure 3 Localization of Sans (green) in the cochlear hair cells of *Ush1c*^{−/−} mice at PD21. Cross sections of the organ of Corti of *Ush1c*^{+/−} (a and c) and *Ush1c*^{−/−} (b and d) mice were stained with an antibody to Sans (green). c and d are higher magnification images of a and b respectively. Arrowheads and arrows indicate inner hair cells and outer hair cells (OHC) respectively. Nuclei were stained by DAPI (Blue). Sans was localized in the stereocilia bundles in *Ush1c*^{+/−} cochlear hair cells at PD21 (c). However, in *Ush1c*^{−/−} mice, strong signals were observed towards the base of stereocilia close to their insertion into the cuticular plate with a slight cytoplasmic staining of OHC (d). Bars indicate 20 μ m.

Ush1c^{−/−} mice, characterized by a shift of the immunoreactivity of the proteins towards the base of stereocilia.

Discussion

The USH gene products are part of a protein complex in hair cells of the inner ear. The actin-bundling and PDZ-domain-containing protein harmonin may coordinate the activities of the USH proteins and bridge them to the cytoskeleton of the hair cell (Boeda *et al.* 2002). Disruption of the USH protein network leads to stereociliary disorganization, as observed in mouse models, and is thought to be responsible for congenital deafness in patients with USH (Petit 2001).

Mouse models for USH have played a crucial role in identifying defective genes responsible for USH1 in humans and furthering our understanding of the function of USH1 proteins in normal and disease conditions. All mouse USH1 models are deaf and exhibit vestibular dysfunction. In these mutants, the sensory cells of the cochlea display anomalies in hair bundle development, indicating an essential function for USH1 proteins in stereocilia differentiation (El-Amraoui & Petit 2005). The abnormal stereocilia morphology

observed in our *Ush1c* knockout mice is similar to that reported in mouse models for other forms of human USH1.

Regarding spatiotemporal expression, immunohistochemical studies show that the USH1 proteins are expressed in hair cells of the inner ear throughout life. However, USH1 protein subcellular distribution in the stereocilia varies dramatically during development until maturity is reached. Expression of harmonin, Cdh23 and Pcdh15 is detectable in the hair bundle from the moment the bundle emerges at the apical surface of sensory hair cells (Boeda *et al.* 2002; Ahmed *et al.* 2003). Harmonin b is found concentrated at the tips of stereocilia during early postnatal stages but its expression diminishes around PD30 in both the cochlea and vestibule (Boeda *et al.* 2002). The spatiotemporal expression pattern of Cdh23 parallels that of harmonin b, being first observed along the entire length of the emerging stereocilia and then restricted to the tip region.

Notably, Grillet *et al.* (2009) have recently shown that harmonin b is a component of the upper tip-link density, where CDH23 inserts into the stereociliary membrane and is required for normal hair cell mechanoelectrical transduction. In foetal cochlea, Pcdh15 can be detected in supporting cells, outer sulcus cells and the spiral ganglion (Alagramam *et al.* 2001b), while in the mature inner ear, Pcdh15 is also localized in stereocilia of sensory hair cells of both the cochlea and the vestibular organ (Ahmed *et al.* 2003). CDH23 and PCDH15 have been shown to be present in the transient lateral stereocilia and kinocilia links and that the two cadherin proteins interact to form tip-link filaments in sensory hair cells (Sollner *et al.* 2004; Michel *et al.* 2005; Kazmierczak *et al.* 2007). MYO7A is expressed in the mechanosensory hair cells of the vestibular organ and cochlea where it is predominantly localized in the stereocilia, but is also detected within the cuticular plate and the pericuticular necklace region, which is characterized by a dense ring of vesicles (El-Amraoui *et al.* 1996; Hasson *et al.* 1997; Boeda *et al.* 2002).

In this study, we investigated the effect of the *Ush1c* knockout mice on subcellular expression of Myosin 7a, Pcdh15 and Sans in the inner ear. We observed the same distribution of Myosin 7a expression throughout the cytoplasm in knockout and control mice which may indicate that Myosin 7a is expressed earlier than harmonin. This may also suggest that Myosin 7a does not rely on the presence of harmonin isoforms for its cytoplasmic distribution. Whether the cytoplasmic Myosin 7a requires harmonin for hair cells function, however, remains to be examined. We detected Pcdh15 at the base of stereocilia and in the cuticular plate in cochlear hair cells from *Ush1c*^{+/−} controls, whereas in the mutant *Ush1c*^{−/−}, Pcdh15 immunoreactivity was found accumulated in the apical region of the OHC and no defined staining was detected at the base of stereocilia nor in the cuticular plate. The scaffold protein Sans has previously been shown localized in the apical hair cell bodies underneath the cuticular plate of cochlear and vestibular hair cells of PD3 mice (Adato *et al.* 2005), but not in the stereocilia. Using an antibody against a peptide sequence corresponding

to the central portion of Sans (amino acid 354–372), we found the protein localized in the stereocilia bundles of mouse cochlear hair cells at PD21 in controls mouse. However, in cochleae from *Ush1c*^{-/-} mice, strong Sans signals were observed towards the base of stereocilia close to their insertion point into the cuticular plate with a slight staining of the cytoplasmic region of OHC. Overall, our data indicated that in mice deficient in harmonin, both interacting partners Pcdh15 and Sans are mislocalized. The epitopes recognized by our antibodies against Pcdh15 and Sans were shifted towards the basal body of the hair cells, whereas they are expressed in the stereocilia of normal control mice.

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